The Influence of Ectotrophic Mycorrhizal Fungi on the Resistance of Pine Roots to Pathogenic Infections II. Production, Identification, and Biological Activity of Antibiotics Produced by Leucopaxillus cerealis var. piceina

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ABSTRACT

An antifungal and antibacterial antibiotic produced by the ectotrophic mycorrhizal fungus *Leucopaxillus cerealis* var. *piceina* has been identified as diatretyne nitrile, using biological and spectrophotometric techniques. Maximum production of this antibiotic occurred during the rapid and incipient autolysis growth phases in liquid medium. During continued autolysis the nitrile was reduced to diatretyne amide and diatretyne 3, which are antibacterial but not antifungal substances.

Concentrations causing minimum inhibition (20%) to germination of zoospores of *Phytophthora cinnamomi* were 50-70 parts/billion (ppb), with total (100%) inhibition at 2 parts/million (ppm). Approximately half of the inhibited zoospores germinate upon removal (dialysis) of diatretyne nitrile between these concentrations, indicating a fungistatic effect. Zoospores failed to germinate after removal of higher concentrations, indicating that these concentrations were lethal. Concentrations causing minimum inhibition (20%) to growth from mycelial discs of *P. cinnamomi* were detected at 0.5 ppm, with 100% inhibition at 9 ppm. Soil bacteria were inhibited between 0.5 and 2.5 ppm. The fungus produced approximately 12 ppm of diatretyne nitrile in liquid culture after 50 days' growth at 15 C. Diatretynes were produced in a variety of aseptic organic substrates to which sucrose or malt extract was added.

Diatretyne nitrile did not inhibit germination of aseptic shortleaf pine seeds exposed for 1 or 2 hr to concentrations up to 40 ppm, but it was phytotoxic to aseptic seedlings at concentrations exceeding 10 ppm. Phytopathology 59:411-417.

Antibiotic production by the ectotrophic mycorrhizal fungus *Leucopaxillus cerealis* var. *piceina* (Peck) (syn. *Clitocybe piceina* Peck, *L. albissimus* var. *piceinus* [Pk.] Singer and Smith, and *L. cerealis* Sing.) was described in the first paper of this series (10). The fungus produced antifungal and antibacterial substance(s) in a variety of agar and liquid media.

In preliminary characterization studies (9) using a bioassay involving germination of zoospores of *Phytophthora cinnamomi* Rands (10), the antibiotic principle in culture filtrates of the fungus was found to be dialyzable. The active dialysate lost approximately one-third of its activity upon evaporation to dryness at 70-100 C in vacuo for 40 to 60 hr. Adjustments of filtrates to reactions of pH 3.0 to 5.0, with 1 N HCl or 1 N NaOH, increased antibiotic activity over that of pH 6.0 by nearly one-third. Antibiotic activity was not found at pH 6.5 or higher. Germination of zoospores of *P. cinnamomi* was optimal in filtrates of *Pisolithus tinctorius* (Pers.) Coker and Couch, another mycorrhizal symbiont used for comparison, and in the control medium between pH 3.0 and 8.0. Zoospores did not germinate in any filtrate adjusted to pH 2.0 or 9.0. Filtrates adjusted to pH 3.0, 4.0, or 5.0 retained activity during 32 days' incubation at 20 and 25 C. This latter finding indicates that the loss of antibiotic activity in older cultures of *L. cerealis* var. *piceina*, as reported in the earlier paper (10), depended upon direct metabolic activity of the fungus, rather than upon physical decomposition of the antibiotic at various temperatures or increasing acidity of liquid cultures.

Anchel et al. (4) reviewed work on a unique group of polyacetylenic antibiotic substances, called diatretynes, from culture filtrates of several hymenomycetous fungi. *Clitocybe diatreta* (Fr.) Kumm., *C. rivulosa* (Pers. ex Fr.) Kumm., *C. odora* (Bull. ex Fr.) Kumm., and *Lepista nuda* (Bull. ex Fr.) Cooke produce one or more of the diatretyne compounds, and are included in Trappe's list (14) of probable ectotrophic mycorrhizal associates. The most active diatretyne antibiotic, diatretyne nitrile, is both antifungal and antibacterial. Diatretyne amide, a reduction product of the nitrile, is antibacterial, and diatretyne 3, a subsequent reduction product of the amide, is weakly antibacterial.

The biological activity of filtrates of *L. cerealis* var. *piceina* is similar to that reported for diatretyne nitrile, and results of the preliminary characterization studies indicated that the antibiotic produced by the fungus could be diatretyne nitrile. In addition, diatretyne nitrile is produced by Hymenomycetes closely related to *L. cerealis* var. *piceina*, a feature that, together with synthesis of the other diatretynes, has been associated with the taxonomic position of these fungi. The structures of the diatretyne antibiotics (4) follow: HOOC—CH=CH—C≡C—C≡C—C≡N, diatretyne nitrile; HOOC—CH=CH—C≡C—C≡C—$CONH_2$, diatretyne amide; HOOC—CH=CH—C≡C—C≡C—C≡C—CH_2OH, diatretyne 3.

The objectives of this paper are to report the identity of the antibiotic(s) produced by *L. cerealis* var. *piceina*, their biological activity, and the physiology of antibiotic production.

MATERIALS AND METHODS.—*Antibiotic identification.*

—*Leucopaxillus cerealis* var. *piceina* was grown in modified Melin-Norkrans' (MMN) liquid medium at 15 C for 50 days to obtain maximum antibiotic yield (10). Culture filtrate volumes of 350 ml were frozen rapidly in acetone-dry ice mixture, freeze-dried overnight, and stored at 5 C in darkness for a maximum of 12 days. Freeze-drying did not affect the biological activity of the test material.

Following procedures described by Anchel et al. (4) for isolation and identification of diatretyne nitrile, each freeze-dried residue was resuspended in 100 ml distilled water, adjusted to pH 2.0 with concentrated HCl, and extracted three times with ethyl acetate using 50-, 30-, and 30-ml volumes. The ethyl acetate fraction was concentrated to approximately one-half its volume (50-55 ml), and extracted twice with equal volumes of 5% aqueous sodium bicarbonate. The bicarbonate solution was extracted with ethyl acetate using 50-, 30-, and 30-ml volumes. Traces of water in the ethyl acetate fraction were removed with anhydrous sodium sulfate. The water-free solvent fraction was filtered through alcohol-washed filter paper, and concentrated in vacuo at 30 C to approximately 2.5-3 ml. This fraction, upon transfer to water (pH 5.0) and in vacuo evaporation of the ethyl acetate, was extremely active against zoospores at half the concentration of the original filtrate. The aqueous phase, the first ethyl acetate fraction, and the bicarbonate fraction were inactive at 8 to 10× concentrations of the original filtrate, which indicated that all antibiotic activity was in the final ethyl acetate fraction. All fractions of the control medium gave negative zoospore bioassays.

A small sample (approximately 0.01 mg) of the ethyl acetate residue was placed in 5 ml of 95% ethyl alcohol, and the ultraviolet (UV) absorption spectrum was determined between 200 and 350 mμ with a Bausch and Lomb Spectronic 505 Spectrophotometer. The infrared absorption spectrum was determined with a double-beam, I-R spectrophotometer by spreading approximately 2 mg of the ethyl acetate residue over a NaCl cell as a liquid film.

Acid hydrolysis of the extract was performed by suspending 0.5 g of the ethyl acetate residue in 50 ml of a 1:1 volumetric mixture of concentrated sulfuric and acetic acids, and incubating at 70 C for 16 hr. After dilution to 200 ml with water, the suspension was extracted for the diatretynes and concentrated for UV spectral analysis.

Biological comparisons of diatretyne nitrile and culture extracts.—The ethyl acetate extract of *L. cerealis* var. *piceina* was evaporated in vacuo at 30 C until the UV absorption maxima at 303 and 323 mμ wavelengths were of the same magnitude as those of the purified diatretyne nitrile furnished by M. Anchel, New York Botanical Garden, Bronx Park. In this manner, i.e., by applying Beers' Law (13), both test solutions were standardized at 1,000 ppm, after which assay solutions of various concentrations were prepared at pH 5.0 in 0.05 M phosphate buffer (15). Ethyl acetate extracts of control medium at the same concentrations were also tested. Zoospore germination, vegetative growth of *Phytophthora cinnamomi*, and bacterial bioassays were used as described previously (10). All bioassays were performed in triplicate at pH 5.0, and each test was repeated.

Pattern of diatretyne synthesis at different phases of growth.—Flon and Anchel (6) demonstrated that diatretyne production in liquid cultures of *Clitocybe diatreta* usually began with either diatretyne nitrile or diatretyne 3, and diatretyne amide normally appeared later. They also found that the total UV absorption maxima of the polyacetylenes tended to disappear with time. This pattern of synthesis and degradation could explain the rapid loss of biological activity in 50-day liquid cultures of *L. cerealis* var. *piceina* grown at 25 C that were highly active at 40 days of age (10). Diatretyne nitrile could have been reduced to either the amide or diatretyne 3, which are not antifungal, shortly after 40 days, thus accounting for the loss of antifungal activity with a longer retention of antibacterial activity in older cultures. A study was designed to follow, spectrophotometrically and biologically, the pattern of diatretyne production in liquid cultures of *L. cerealis* var. *piceina* grown for extended periods.

Leucopaxillus cerealis var. *piceina* was grown in MMN liquid medium at 25 C. At 10-day intervals, for 90 days, mycelium was harvested and culture filtrates were obtained. Twenty-five ml of each filtrate were Millipore filter-sterilized and reincubated aseptically at 25 C in darkness. These samples were tested for antibiotic activity by the zoospore germination bioassay at 10-day intervals to determine if incubation of active filtrates at 25 C reduced antibiotic activity.

A 160-ml volume of each filtrate was adjusted to pH 2.0 and extracted with ethyl acetate, sodium bicarbonate, and again with ethyl acetate. The final ethyl acetate volume was divided into two equal volumes and concentrated in vacuo at 30 C to 3 ml each. One sample was diluted to 80 ml in 0.05 M phosphate buffer (pH 5.0), reconstituting it to the original filtrate concentration. The ethyl acetate was removed from this volume in vacuo at 30 C. This suspension, at several dilutions, was used in the zoospore bioassay. The remaining 3-ml residue was suspended in 95% ethyl alcohol for determination of the UV absorption spectrum. Control MMN liquid medium was extracted and resuspended as above for UV spectral and biological comparisons.

Standard graphs for absorption and concentration were made for known amounts of purified diatretyne nitrile, diatretyne amide, and diatretyne 3 in alcohol for various wavelengths in the UV spectrum. A small amount of control medium-ethyl acetate concentrate was added to each of these pure diatretyne alcohol solutions to furnish background absorbancy similar to the filtrate extract spectra. The absorption maxima used for quantitative determinations of the diatretynes were 285, 303, and 323 mμ for the nitrile; 260 and 275 mμ for the amide; and 340 mμ for diatretyne 3.

These data were compared to those from the various filtrates to determine the relative amounts of each diatretyne compound present in the different *Leucopaxillus cerealis* var. *piceina* filtrate extracts. After cal-

calation of the approximate amounts in each culture filtrate extract, UV spectra were made of alcohol solutions of mixtures of the pure diatretynes calculated as being present in the filtrate extract. In order to duplicate the UV spectra, it was necessary to modify slightly the amounts of each diatretyne present. When reasonable duplication of the filtrate spectrum was completed, the formulated diatretyne mixture was tested for similarity of its biological activity to that of the respective filtrate, using the zoospore germination bioassay. The results from the zoospore assays did not require reformulation of any diatretyne combination.

Production in supplemented natural substrates.—Two hundred and fifty ml of screened shortleaf pine humus collected from 02 horizon of soil under 15- to 18-year-old shortleaf pine trees near Raleigh, North Carolina, and 150 ml of either (i) complete Melin-Norkrans' (MN) nutrient solution (12), (ii) complete MMN liquid medium (10), (iii) these media with certain ingredients removed, or (iv) sucrose at various concentrations were added to 2-quart Mason jars. The jars were plugged with cheesecloth-reinforced cotton, and autoclaved for 1 hr at 121 C. Reactions of all substrates after autoclaving was pH 3.8 to 4.1. Four jars/treatment were seeded (10) with *L. cerealis* var. *piceina*, and two jars/treatment were retained as noninoculated controls. After 90 days at 25 C the relative growth of *L. cerealis* var. *piceina* was estimated visually.

Diatretyne compounds were extracted from 50 g (wet wt) from each of the four replicate jars of treatments in which the fungus exhibited visible signs of growth. Samples of 100 g from each of the 2 control jars were removed. Treatment samples of 200 g each were blended for 3 min in an electric blender with 1 ml of 0.012 N HCl, and stored at 5 C. After 18 hr the samples were centrifuged at 2,000 rpm for 30 min at 2 C, and the solution filtered twice through Whatman No. 1 filter paper over a Büchner funnel with slight vacuum. Reactions of all leachates were pH 2.0. Ethyl acetate and sodium bicarbonate fractionations were made, and UV spectra determined.

Production in substrates of different acidity.—Nine hundred ml of vermiculite and peat moss were added to 2-quart Mason jars in ratios previously reported [11] for reactions of pH 4.0, 4.6, and 5.6; pure vermiculite was used for pH 6.6. Five hundred ml of MN nutrient solution were added to each jar. The jars were autoclaved for 30 min at 121 C. Four jars/acidity level were seeded with *Leucopaxillus cerealis* var. *piceina*, and two noninoculated jars served as controls. After 90 days' incubation at 25 C in darkness, the relative growth of *L. cerealis* var. *piceina* was estimated visually. Substrate samples were removed, extracted, and UV spectral analyses were performed.

Production in shortleaf pine root extract medium.—Shortleaf pine seeds were surface-sterilized in 30% H_2O_2 for 30 min, planted in 4-inch plastic pots filled with autoclaved vermiculite:sand mixture (1:1, v/v), and incubated in the growth chamber with 2,500 ft-c light for a 14-hr photoperiod at 23 C. Night temperatures were 20 C. Each pot was moistened alternately with sterile, distilled water and MN nutrient salts twice a week. After 4 months, the substrate was washed from the roots. The roots were excised at the collar region, placed in polyethylene bags, and frozen at −18 C. After 2 weeks, the roots were thawed, and one-hundred g (wet wt) of unsuberized roots (both short and lateral root tips) were removed and minced in 400 ml of MN nutrient solution in an electric blender for 10 min. The suspension was mixed vigorously, using magnetic stirring, at 5 C for 12 hr. The cell debris was removed by filtering once through filter paper and three times through Seitz C-2 clarifying filters. The filtered volume was brought to 1 liter with MN nutrient solution to obtain a 10% root extract concentration. The control consisted of MN nutrient solution without root extract. After adjustment of reaction to pH 5.0 with 1 N HCl, both media were Millipore filter-sterilized twice. Volumes of 100 ml were pipetted aseptically into 250-ml Erlenmeyer flasks. *Leucopaxillus cerealis* var. *piceina* was seeded into six flasks of both the root extract and control media; 4 control flasks/medium remained free of the fungus. Three flasks with *L. cerealis* var. *piceina* were removed after 35 and 55 days of growth at 25 C, and the mycelium harvested and weighed, as were two control flasks from each medium. Volumes of 150 ml of culture filtrate and of control media were extracted for detection of the diatretynes. The ethyl acetate residues were used for UV spectral analysis and for zoospore bioassay. Quantitative determinations of the diatretyne compounds were made, using zoospore assay and graphs relating concentration with UV absorbancy of the various diatretynes.

Phytotoxicity studies with shortleaf pine.—To determine the influence of diatretyne nitrile on germination, shortleaf pine seeds were surface-sterilized in 30% H_2O_2 for 30 min, and soaked at 25 C for 3 hr each in four changes of 40 ml sterile, distilled water/100 seeds. Approximately 200 seeds were soaked in 30 ml diatretyne nitrile in 125-ml Erlenmeyer flasks at concentrations between 1 and 40 ppm in 0.05 M phosphate buffer at a reaction of pH 5.0. Control seeds were soaked in 30 ml phosphate buffer only. All flasks were shaken vigorously by hand at 15-min intervals. Approximately 100 seeds were removed from solution after 1 and 2 hr, soaked for 5-10 min in 40 ml sterile water, and planted in petri plates containing 1% corn meal with dextrose agar (Difco). The plates were then incubated in a growth chamber, and germination percentages determined after 18 days.

To determine the effect of diatretyne nitrile on seedling growth, Pyrex glass test tubes (130 × 20 mm) were filled to a depth of 4 cm with 4-mm-diam, acid-washed glass beads and 3 ml of the salts of MN nutrient solutions in 0.05 M phosphate buffer at a reaction of pH 5.0. After autoclaving for 10 min at 121 C, 0.5 ml of various concentrations (0-40 ppm) of diatretyne nitrile in phosphate buffer (pH 5.0) were added. Aseptically germinated shortleaf pine seeds with radicles 2-3 cm in length were planted in eight replicate tubes/diatretyne concentration. An additional three tubes/concentration remained free of seedlings. The latter

tubes were used to determine the relative amounts of the antibiotic present after incubation in the absence of seedlings. All tubes were incubated in the growth chamber, and re-randomized every other day. After 40 days, all seedlings were removed, and height, color, and radicle development recorded.

Each tube was filled with 20 ml 0.012 N HCl cooled to 5 C, shaken vigorously for 10-15 min, and the liquid decanted from the bead substrate. The reaction of these solutions was pH 2.4 to 2.7, and each was adjusted to pH 2.0 with 1 N HCl. Volumes of 150-170 ml and 35-45 ml were obtained from the treatments with and without seedlings, respectively. Approximately 100-160 mg of seedling foliage from the 0, 0.5, 2, 6, and 10 ppm diatretyne nitrile treatments were minced with mortar and pestle in 20 ml 0.012 N HCl at 5 C. Cell debris was removed by filtration, and all filtrates were adjusted to pH 2.0. After storage at 5 C for a maximum of 48 hr, all solutions from the tubes and foliage were extracted for diatretyne nitrile, and the UV spectra determined.

RESULTS.—*Antibiotic identification.*—The UV absorption spectrum of the alcohol solution of the extracted filtrate was identical to that of diatretyne nitrile (2). No traces of diatretyne amide (1) or diatretyne 3 (3, 6) were evident (Table 1). The UV spectra from extracts of several freeze-dried replicates of *L. cerealis* var. *piceina* extract were identical. Control extracts absorbed slightly between 220 and 330 mμ without maxima at any specific wavelength.

The results of infrared analysis indicated the presence of acetylenic linkages (R—C≡C) at 2,000 cm^{-1} and nitrile (R—C≡N) at 2,235 cm^{-1}. An absorption at 1,690 cm^{-1} was observed also, and was attributed to a carbonyl linkage from an ester or acid (R—CO—R); it was not associated with an amide linkage (R—$CONH_2$) because conjugated amides usually absorb at lower frequencies (5). This analysis was repeated several times, using different samples with similar results. Control extracts were negative for these absorption maxima.

Following acid hydrolysis of the active extract and extracting for the diatretynes, a UV spectrum characteristic of diatretyne amide was obtained. This test was repeated three times, with the same results. Control extracts were negative for specific absorption maxima. Anchel (2) reported that diatretyne nitrile after acid hydrolysis was transformed into diatretyne amide using this technique.

TABLE 1. Ultraviolet light absorption maxima of an ethyl acetate extract from culture filtrates of *Leucopaxillus cerealis* var. *piceina* and maxima of diatretyne nitrile, diatretyne amide, and diatretyne 3

Absorption maxima at indicated wavelengths (mμ) from:			
Extract of *L. cerealis* var. *piceina*	Diatretyne nitrile	Diatretyne amide	Diatretyne 3
230	230	224	253
240	239	260	280
254	254	274	297
269	268	291	316
285	285	310	339
303	303		
323	322		

Biological comparisons of diatretyne nitrile and culture extracts.—Both test substances at each concentration (0.01 to 30.0 ppm) had almost identical effects in the bioassays. The minimum inhibitory concentration (MIC) to *P. cinnamomi* zoospore germination, i.e., concentration causing a 20% reduction in germination, was 50-70 parts/billion (ppb), and the absolute inhibitory concentration (AIC), i.e., concentration causing 100% inhibition to germination, was 2 ppm. Concentrations of diatretyne nitrile between these extremes were fungistatic, and concentrations above 2 ppm were lethal to zoospores. Fungistasis was demonstrated by exposing zoospores for 8 hr to different concentrations of diatretyne nitrile, followed by overnight removal of the antibiotic by dialysis at 5 C. Thirty to 50% of previously inhibited zoospores exposed to the antibiotic between the MIC and AIC germinated upon incubation at 23 C for 2-3 hr. Zoospores exposed to 2-9 ppm of the antibiotic failed to germinate upon its removal, indicating that these concentrations were lethal.

The MIC to growth of *P. cinnamomi* from mycelial discs in MMN liquid medium, i.e., causing a 20% growth reduction as compared to controls, was 0.5 ppm. The AIC was 9 ppm. This concentration was fungistatic since vigorous growth from these discs occurred after 48-hr incubation at 23 C on V-8 juice agar medium (10).

The MIC to various soil bacteria, i.e., concentration causing a 20% reduction in number of bacterial colonies after incubation with the antibiotic in 3% sucrose solutions, was 0.5 ppm, and the AIC, i.e., concentration causing 100% reduction in number of bacterial colonies, was 2.5 ppm. Control medium extracts were negative in all bioassays.

Concentrations of diatretyne nitrile, *L. cerealis* var. *piceina* extract, and control medium extract of 0.1, 0.5, and 2 ppm adjusted to pH 2.0 to 8.0 with 1 N HCl or 1 N NaOH were prepared from the original unbuffered 1,000-ppm samples. Both the *L. cerealis* var. *piceina* extract and diatretyne nitrile strongly inhibited zoospore germination between pH 3.0 and 5.0, with slight loss of biological activity at pH 6.0. Very little activity was detected at pH 7.0 or 8.0. The control medium extract did not inhibit germination above pH 3.0. These results were almost identical to those from preliminary studies on antibiotic characterization of culture filtrates of *L. cerealis* var. *piceina* at different pH reactions.

On the basis of these analytical and biological tests, it was concluded that the antibiotic principle in filtrates of *L. cerealis* var. *piceina* was diatretyne nitrile. This conclusion was confirmed by Anchel (*personal communication*) after examination of the data. After 50 days at 15 C in MMN liquid medium, *L. cerealis* var. *piceina* produced approximately 12 mg of diatretyne nitrile/liter of filtrate.

Pattern of diatretyne synthesis at different phases of growth.—Production of diatretyne nitrile increased

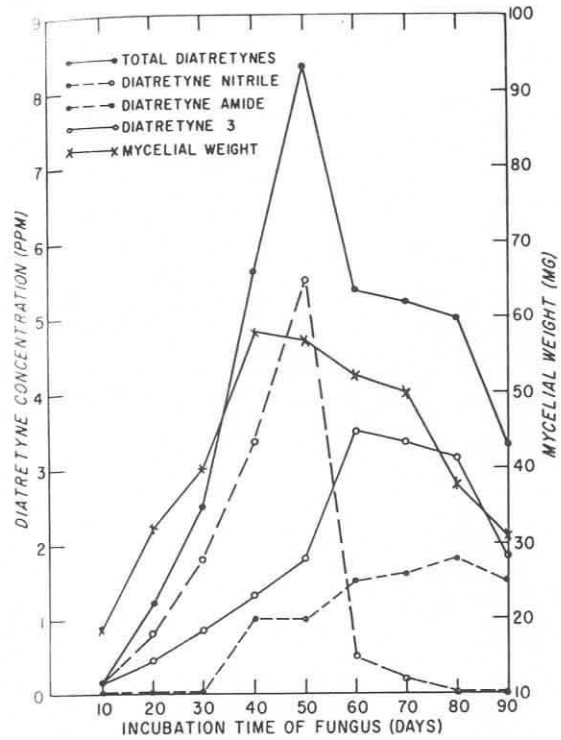

Fig. 1. Pattern of diatretyne synthesis and degradation by *Leucopaxillus cerealis* var. *piceina* in Melin-Norkrans' (MMN) liquid medium during 90 days' incubation at 25 C.

with growth of *Leucopaxillus cerealis* var. *piceina* (Fig. 1). The diatretyne nitrile concentration increased to a maximum (5.5 ppm) in 50-day cultures. This quantity of nitrile, however, was lost rapidly during autolysis, and the 60-day filtrate had less than 10% (0.4 ppm) of the concentration present in 50-day series. The amount of diatretyne nitrile continued to decrease, until at 80 days it was not detectable. Diatretyne 3 was detected first in the 20-day culture, and gradually increased in concentration during the rapid and autolytic growth phases (Fig. 1). Its greatest increase was between 50 and 60 days, the same period during which the nitrile concentration sharply decreased. Diatretyne 3 gradually decreased in concentration with age. Diatretyne amide was not detected in the filtrates until after 40 days (Fig. 1). It probably was present at low concentrations in the younger cultures, but could not be detected with the analytical procedures employed. The detectable amounts of the amide, with increasing incubation time, generally followed the pattern of diatretyne 3.

The total concentration of all the diatretynes at different ages corresponded to that reported by Flon and Anchel (6). That is, the diatretynes tended to decrease in quantity with increasing incubation. This decrease of the total concentration of the diatretynes and the rapid decrease in concentration of diatretyne nitrile after 50 days was ascribed to the metabolic activity of *L. cerealis* var. *piceina* and not to physical factors. Results from zoospore assays of the sterile filtrates incubated at 25 C supported this conclusion. The most active filtrates from 40- and 50-day cultures at pH 4.1 and 3.9, respectively, did not noticeably lose activity after an additional 50 days' incubation at 25 C.

Production in natural supplemented substrates.—*Leucopaxillus cerealis* var. *piceina* produced diatretyne nitrile and diatretyne 3 in all natural substrates tested in which it grew (Table 2). The amide was detected only in those substrates in which the fungus grew vigorously. All control treatments and those treatments not supporting growth of the fungus were negative for diatretyne chromophores.

Sucrose and malt extract supplements were of major importance in growth of the fungus and diatretyne production. Apparently native carbon was not available in significant quantities in the humus, because the addition of MN salts and thiamine without carbon sup-

TABLE 2. Diatretyne production by *Leucopaxillus cerealis* var. *piceina* in sterile supplemented shortleaf pine humus after 90 days at 25 C

Basic supplements	Supplement, modifications	Visual growth of *L. cerealis* var. *piceina*	Diatretyne compounds detected[a]
Melin-Norkrans' nutrient (MN) solution	MN complete	Moderate	DN, D3
	MN w/o thiamine (1)	Moderate	DN, D3
	MN w/o sucrose (2)	None	0
	MN w/o (1) and (2)	None	0
Modified Melin-Norkrans' medium (MMN)	MMN complete	Vigorous	DN, DA[b], D3
	MMN w/o thiamine (1)	Vigorous	DN, DA[b], D3
	MMN w/o sucrose (2)	Moderate	DN, D3
	MMN w/o malt extract (3)	Moderate	DN, D3
	MMN w/o (1) and (2)	Moderate	DN, D3
	MMN w/o (1) and (3)	Moderate	DN, D3
	MMN w/o (2) and (3)	None	0
Sucrose	0	None	0
	1.0%	Poor	DN[b]
	2.5%	Moderate	DN, D3
	3.0%	Moderate	DN, D3

[a] DN = diatretyne nitrile; DA = diatretyne amide; D3 = diatretyne 3.
[b] Trace amounts detected only.

plement did not support growth of *L. cerealis* var. *piceina*.

Production in substrates of different acidity.—Substrate reaction influenced growth of *L. cerealis* var. *piceina*. The symbiont grew equally well at pH 4.0 to 5.6, but was less vigorous at pH 6.6. Diatretyne compounds were produced by *L. cerealis* var. *piceina* over the acidity range tested. None were detected in the control series at the different reactions. Similar amounts of diatretyne nitrile and diatretyne 3 were present in substrates at pH 4.0 to 5.6, whereas the amounts from substrate of pH 6.6 were considerably lower. This difference may have been the direct effect of pH on synthesis but, more likely, it was caused by the limited growth of *L. cerealis* var. *piceina* at pH 6.6.

Production in shortleaf pine root extract medium.—The fungus grew very well in the root extract medium, and produced a significant quantity of diatretyne nitrile and a lesser amount of diatretyne 3 (Table 3). Autolysis was apparent in the 55-day root extract culture, and diatretyne nitrile concentration decreased sharply. In contrast, growth and diatretyne production in the control medium was slight. The fungus apparently remained in the rapid phase of growth in the control medium at 55 days, since mycelial weight increased after 35 days. Diatretyne amide was not detected in filtrates of either treatment.

Phytotoxicity studies with shortleaf pine.—Diatretyne nitrile did not inhibit shortleaf pine seed germination at the concentrations and exposures tested. Germination percentages of 38-44% were recorded from the diatretyne nitrile treatments, and 39-41% from the control series after 1- and 2-hr exposures, respectively. Time of germination and relative vigor of radicle elongation were not affected by diatretyne nitrile. All seeds initiated germination after 7-8 days, and all radicles were between 2-3 cm in length after an additional 10 days.

Eight germinated seeds from the 2-hr exposure series of the 0, 0.5, 2, 10, 20, 30, and 40 ppm diatretyne nitrile treatments were planted in separate flats of autoclaved sand moistened with MN nutrient salts. After 40 days' incubation in the growth chamber with weekly applications of MN nutrient salt solution, no differences in seedling height, needle color, or root development could be detected.

TABLE 3. Growth and diatretyne production by *Leucopaxillus cerealis* var. *piceina* in Melin-Norkrans' nutrient solution with and without 10% shortleaf pine root extract after 35 and 55 days at 25 C

Medium	Incubation	Mycelial weight	Diatretyne chromophore	Diatretyne concn
	days	*mg*		*ppm*
MN nutrient solution and 10% root extract	35	44.1	Nitrile	4.6
			3	1.5
	55	39.4	Nitrile	1.0
			3	2.5
MN nutrient solution (control)	35	6.2	Nitrile	0.5
			3	0.5
	55	7.8	Nitrile	0.9
			3	0.5

Measurements of the six best seedlings/treatment showed that diatretyne nitrile at high concentrations was phytotoxic to shortleaf pine seedlings. Concentrations of 20 ppm and higher inhibited seedling growth completely, indicating that the phytotoxicity level was between 10 and 20 ppm. Concentrations up to 6 ppm did not adversely affect height growth, needle color, or radicle development, whereas seedlings developed poorly in the 8- and 10-ppm concentrations.

Spectrophotometric UV analysis of the extracts from foliage and from solution from tubes failed to detect diatretyne nitrile. Apparently, the polyacetylene was not stable for 40 days under the test conditions in tube culture, nor was it translocated to the foliage in a stable, detectable form.

DISCUSSION.—The identification of diatretyne nitrile as the antibiotic principle in culture filtrates of *Leucopaxillus cerealis* var. *piceina* adds another hymenomycete to the list of those that produce the diatretyne antibiotics. This fungus was compared physiologically to several hymenomycetes known to produce diatretynes, and sufficient differences were found to conclude that *L. cerealis* var. *piceina* is probably a distinct species (9). This fungus is included in Singer's *Tricholomataceae,* along with the majority of other fungi which produce these antibiotics. This feature, as discussed by Anchel et al. (4), supports Singer's classification system, because most of these fungi belong to genera which Singer places in the *Tricholomataceae.*

The inhibitory effect of diatretyne nitrile on the germination of zoospores of *P. cinnamomi* at concentrations as low as 50-70 ppb suggests that the antibiotic inhibits a major metabolic reaction in the zoospores. Evidence was not found to explain the greater sensitivity of zoospores of *P. cinnamomi,* which were killed in 2 ppm concentrations, as compared to vegetative mycelium that was not killed by concentrations as high as 9 ppm.

The synthesis of diatretyne nitrile during the rapid growth phase, followed by its rapid degradation during autolysis, explains the loss of antifungal activity in cultures incubated for extended periods (10). This finding, in addition to the appearance of gradually increasing concentrations of diatretyne amide and diatretyne 3 with increasing autolysis, supports the conclusions of Flon and Anchel (6) that the total diatretyne concentration produced by *Clitocybe diatreta* tended to disappear with increasing periods of incubation. The rapid growth phase production of the nitrile by *L. cerealis* var. *piceina* has possible ecological significance if this sequence of diatretyne production takes place when the fungus is in mycorrhizal association under natural conditions. Diatretyne nitrile should be produced during the most physiologically active phase of mycorrhizal development, thereby being of great potential value as an inhibitor of fungal pathogens during periods when they are likely to be most active in root infection.

The production of diatretynes by *L. cerealis* var. *piceina* in root extract medium, in various supplemented organic substrates, and in substrates of different acidities, shows that this fungus can probably produce these antibiotics on all substrates supporting growth. An

additional food base other than a basic carbon source was not a prerequisite to antibiotic production. The ecological value of antibiotic production by *L. cerealis* var. *piceina* in respect to its saprophytic and symbiotic existence under natural forest soil conditions must await further research. Theoretically, however, the fate of antibiotics produced by this fungus or other mycorrhizal fungi need not necessarily be similar to the fate of antibiotics produced by saprophytic fungi. These latter fungi produce antibiotics in the immediate substrate or "ecological niche" in which they grow. These restricted sites of antibiotic production apparently are not of major significance in reducing pathogen inoculum potential in other than the immediate site (7, 8). The "ecological niche" of mycorrhizal fungi is the host root that ensures these specialized root parasites of essential metabolites (e.g., carbohydrates, vitamins, etc.) for which they need exert only minimal competitive efforts. Antibiotics produced in this niche should be ideally located for inhibitory effects on pathogens attempting infection of these mycorrhizal, or perhaps even adjacent nonmycorrhizal, roots. These antibiotics should also exert a selective effect upon rhizosphere microorganisms.

The phytotoxicity of diatretyne nitrile, at concentrations in excess of 10 ppm, to shortleaf pine seedlings could create problems in subsequent research with *L. cerealis* var. *piceina*. However, this fungus produced this amount only under the best test conditions, i.e., 50 days' incubation at 15 C in MMN liquid medium. Therefore, this level of phytotoxicity may not be significant. Conditions inimical to the production of this amount of diatretyne nitrile should not be encountered in normal aseptic mycorrhizal synthesis, in other tests employing this fungus with pine seedlings, or in field conditions.

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